mirrorball®
application note

enhanced ELISA workflows no-wash multiplexed fluorescent bead assays

introduction

Enzyme linked immunosorbant assays (ELISA) are the gold standard method to quantify cytokine levels in sera or cell culture supernatants. Several vendors offer high quality, validated reagents to carry out cytokine ELISAs with high precision and accuracy, however, the ELISA approach itself suffers from several inherent technical limitations:

- multiple wash stages are laborious, requiring significant hands-on time
- ELISA offers a limited dynamic range, thus pre-dilution of the sample may be required
- ELISAs cannot be multiplexed to quantify several cytokines simultaneously, thus incurring time, reagent and sample costs.

This tutorial demonstrates the advantages of converting standard colorimetric ELISA kits to a no-wash, bead-based immunosorbant assay (FLISA) format using sol-R™ beads and TTP Labtech's mirrorball fluorescence cytometer. We will show how this approach retains the precision and accuracy of the ELISA assay, but overcomes some of the technical limitations by enabling (i) a no-wash, homogenous assay format, (ii) a wider dynamic range and (iii) multiplexing options for up to four cytokines.

Both the conventional ELISA assay and the bead-based FLISA assay utilise the same capture and detection antibody pairs (Fig 1) supplied in the commercial kit. The orientation of the "immuno-sandwich" in the FLISA assay is inverted with respect to the conventional ELISA, as this setup eliminates assay interference from biotin, which is found in many cell culture supernatants. For signal generation, the ELISA assay utilises a streptavidin-HRP conjugate directed against the biotinylated detection antibody, whereas the FLISA utilises a green-fluorescent secondary antibody directed against the other (non-biotinylated) antibody. The streptavidin-coated sol-R beads are coded with different intensities of a red fluorescent dye, which allows intensity-based gating of mixed bead populations (Fig 2). Using this approach, up to four different cytokine FLISAs assay may be multiplexed in a single experiment.

materials and methods

materials

- ELISA kits for human TNF∝, IL-8 and IL-6 (R&D Systems #DY210, #DY208 and #DY206 respectively). Each kit contains:
  - ELISA capture antibody (mouse-anti-human TNF∝ or IL-8 or IL-6) → this will be used as the detection antibody in the inverted FLISA
  - ELISA biotinylated detection antibody (goat-anti-human TNF∝ or IL-8 or IL-6) → this will be used as the capture antibody in the inverted FLISA
  - recombinant cytokine standard (human TNF∝ or IL-8 or IL-6)
- streptavidin-coated sol-R beads (TTP Labtech 4150-09071)
- AlexaFluor 488 conjugated anti-mouse detection reagent (JIR cat# 115-546-071)

key benefits

TTP Labtech's mirrorball and sol-R coded bead protocols offer:

- **decreased hands-on time, budget and sample saving**
- **faster time to decision**
- **no-wash, process efficient protocols**
- **same accuracy and precision as conventional ELISA**
- **multiplexed capability without reducing assay performance**

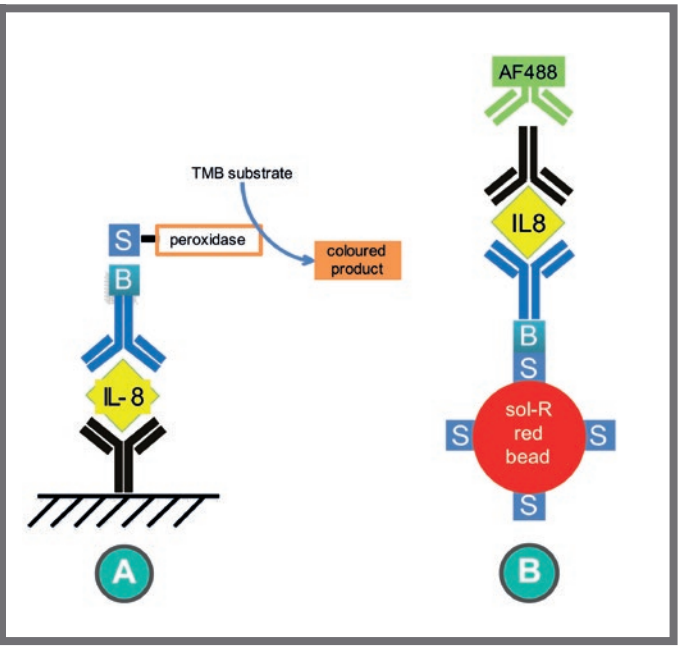

Fig 1. Schematics for conversion of a conventional ELISA assay to an inverted FLISA on beads.

(A) standard ELISA format, where the capture antibody is immobilised onto the polystyrene plate surface and a biotinylated detection antibody is used;

(B) inverted FLISA format, where the biotinylated detection antibody from the ELISA kit is immobilised onto streptavidin-coated bead to act as a capture antibody and the unlabelled capture antibody from the ELISA kit acts as the detection antibody.

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methods

FLISA on the mirrorball fluorescence cytometer

1. coat 2 x 10^5 of the sol-R beads with 2 µg/mL of biotinylated goat-anti-human cytokine antibody according to the user information sheet supplied with the beads.

   Any of the individual fluorescent sol-R bead codes are suitable for single plex experiments, but we recommend using a combination of sol-R2, sol-R4 and sol-R5 for a 3-plex experiment

2. **For the single plex experiments only**: Spin down the coated beads and re-suspend in 0.5 mL PBS, 1% BSA (bead count: 2 x 10^5 beads in 500 µL)
3. **For three-plex experiments only:** Combine the 3 different coated beads in a single tube, spin down, then resuspend the mixture in 0.5 mL PBS, 1% BSA (total bead count: 6 x 10^5 coated beads in 500 µL)
4. to each tube of coated beads, add mouse-anti-human cytokine
5. add 10 µL of recombinant cytokine standard to the wells of a 384-well plate
6. to this, add 10 µL per well of detection mixture diluted in PBS, 1% BSA
7. incubate at room temperature in the dark for 4 hours
8. scan on mirrorball

colorimetric ELISA experiments

Colorimetric ELISA experiments were carried out according to manufacturer instructions.

detection mixture (single plex)	detection mixture (multiplex)
5 x 10^4 coated beads/mL *prepared in previous step*	1.5 x 10^5 coated beads/mL *prepared in previous step*
0.1 µg/mL mouse detection antibody	0.1 µg/mL mouse detection antibody for each cytokine to be tested *i.e.* *0.1 µg/mL of mouse-anti-TNFα* *0.1 µg/mL of mouse-anti IL-8* *0.1 µg/mL of mouse-anti IL-6*
0.2 µg/mL Alexa Fluor 488-conjugated anti-mouse detection antibody	0.2 µg/mL Alexa Fluor 488-conjugated anti-mouse detection antibody

Table 1. Composition of detection mixtures for FLISA assays

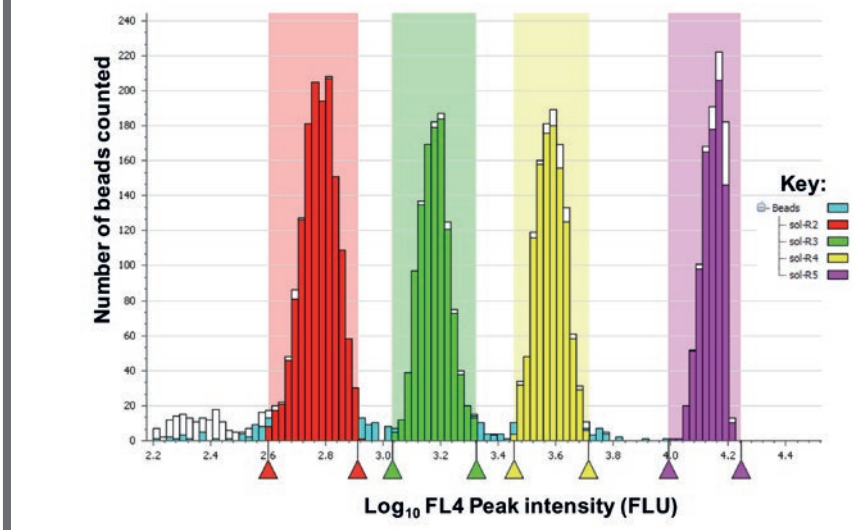

Fig 2. De-coding sol-R beads in the red channel of mirrorball. sol-R2, sol-R3, sol-R4 and sol-R5 beads are shown in red, green yellow and purple respectively

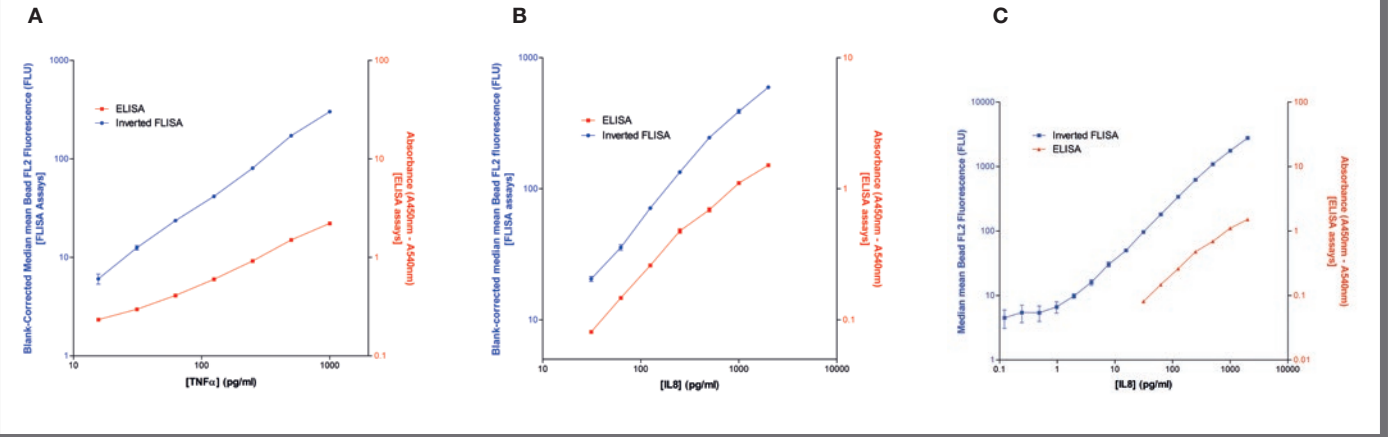

Fig 3. Comparison of single-plex colorimetric washed ELISA with no-wash FLISA. Results are shown for TNFα (A) and IL-8 (B). quantification respectively. Colorimetric washed ELISA data are plotted against the right y axes (red) and no-wash FLISA data are plotted against the left y axes (blue). The absolute lower limit of detection for the IL-8 standard is shown in panel (C).

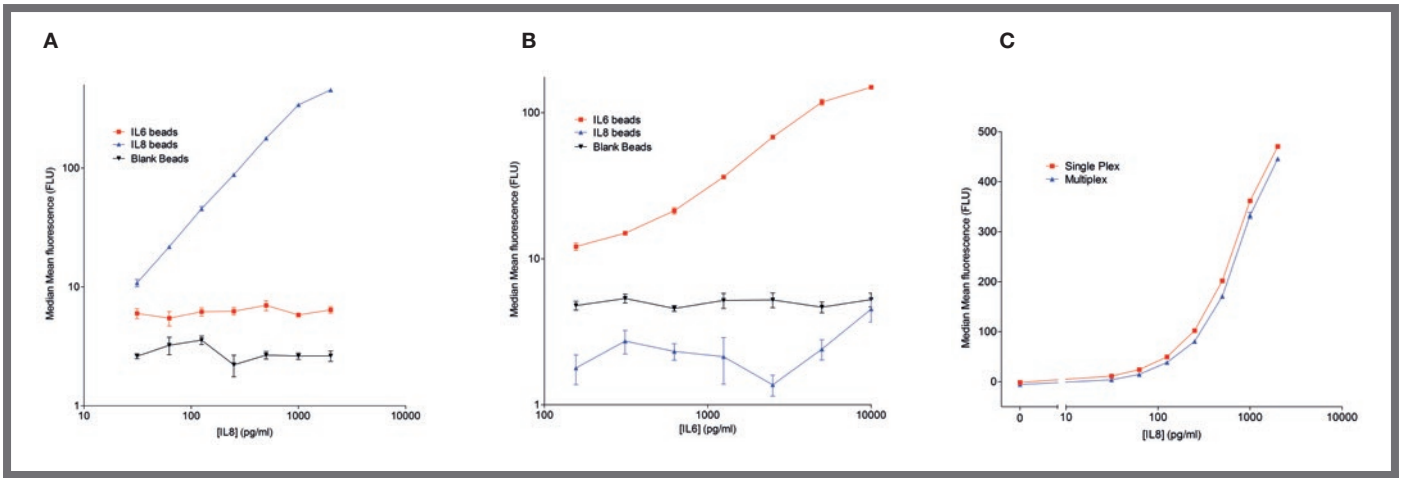

Fig 4. Multiplexed no-wash FLISA. Only the appropriately-coated beads detect the cytokine concentration curve for IL-8 (A) or IL-6 (B) and there is very little difference between carrying out the IL-8 assay in either single plex or multiplex (C). Data plotted are from quadruplicate wells.

results

benchmarking of ELISA and FLISA assay formats

In order to establish if a bead-based, homogenous FLISA assay would generate equivalent data to the conventional ELISA, the two different formats were compared using two representative cytokines: TNFα and IL-8. Fig 3 confirms that both approaches generate equivalent, linear signals against the cytokine standards in the concentration range specified in the kits, with tight error bars confirming the excellent precision of all measurements.

limit of detection determination for the FLISA approach

Next we sought to explore the absolute limit of detection for the homogenous FLISA approach, using the IL-8 FLISA as an example. The conventional ELISA using the commercial kit is validated in the concentration range 31.25-2,000 pg/mL. Our results show that the FLISA limit of detection is 5 pg/mL, extending the FLISA limit of detection and dynamic range by an order of magnitude compared to the ELISA (Fig 3).

multiplexed FLISA performance

Having shown that conventional ELISAs can be readily converted to no-wash FLISA assays, the effect of multiplexing different targets (IL-6 and IL-8) within single wells was investigated. Here, three individual sol-R bead codes are coated with either anti IL-6, anti IL-8 or blank, and combined ahead of dispensing onto a 384-well plate. Figure 4A and B shows that both IL-6 and IL-8 show concentration-dependent binding with their cognate cytokine, with no significant non-specific binding, nor cross reactivity. Assay performance is very similar in both single plex and multiplex (Fig 4). This result demonstrates that it is possible to combine multiple tests within single wells using sol-R beads in a no-wash assay format.

discussion

These data demonstrate that reagents from commercially available multistep ELISA kits can be easily converted into streamlined no-wash multiplexed FLISA protocols with the following advantages over standard ELISA:

- no-wash, process efficient protocols to save hands-on time, samples and reagents
- same accuracy and precision compared to conventional ELISA assay; lower limit of detection and extended dynamic range
- multiplexing capability without reducing assay performance
- easy automation to increase throughput

about mirrorball

The mirrorball plate-based fluorescence cytometer uses TTP Labtech's laser scanning technology to provide HTS-friendly workflows that deliver gold standard data quality. The mirorball's proprietary background rejection optics enable the use of streamlined no-wash assay formats for multiplexed cell-, or bead-based applications that provide process efficiencies over standard ELISA.

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